THE JOURNAL OF INVESTIGATIVE DERMATOLOGY
Vol. 52, No. 3

Printed in U.S.A.

TRIGLYCERIDE HYDROLYSIS BY *CORYNEBACTERIUM ACNES IN VITRO**

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The pathogenetic factors in acne vulgaris, an inflammatory process involving the pilosebaceous units of human skin, have not been defined although the free fatty acids have been implicated by indirect evidence. For example, the intradermal injection of human skin surface lipid produces an inflammatory reaction and a histologic picture resembling acne vulgaris (1). The free fatty acid fraction of human skin surface lipid has been shown to be primarily responsible for this reaction (1, 2).

These fatty acids are incorporated into the triglycerides synthesized by the human sebaceous gland and are liberated in the sebaceous duct and on the skin surface by the hydrolyzing action of bacterial or endogenous enzymes. The microorganism, *Corynebacterium acnes* (*C. acnes, Propionibacterium acnes*) has been suggested as the most likely source of bacterial enzymes (3). Direct evidence is not available, however, regarding ability of this organism to hydrolyze triglycerides of long and short chain fatty acids. To test the hypothesis of fatty acid liberation from triglycerides by the hydrolytic action of *C. acnes, in vitro* incubations of fifteen strains of *C. acnes* were done with three representative triglycerides.

MATERIALS AND METHODS

A. Bacteriology. Fifteen unselected isolates of *C. acnes* were obtained from those maintained in culture in the microbiology laboratory of the department of dermatology. The following criteria were used for identification of the micro-organism: thin pleomorphic rod, Gram positive, catalase positive, growing as a tiny, pin point, round to domed, smooth, white colony at 35–36° C. under anaerobic conditions, with minimal or no growth in the presence of air, fermenting glucose, galactose, and fructose without gas formation and liquefying gelatin. The organisms were isolated from either normal human skin or from comedones, pustules or clinically normal skin of patients with acne vulgaris.

B. Lipids. Three series of triglycerides were used in this study. Tricaprylin (C_8 triglyceride), tricaprin (C_{10} triglyceride) or trilaurin (C_{12} triglyceride) were chosen as representative of triglycerides of shorter chained fatty acids, tripalmitin (C_{16} triglyceride) as representative of triglycerides of long chain saturated fatty acids, and triolein (unsaturated C_{18} triglyceride) as representative of triglycerides of long chain unsaturated fatty acids.

Each bacterial strain was incubated with individual triglycerides in screw-topped culture tubes containing 8 ml of thioglycolate medium, and 1 ml of glycerol as emulsifier. (Later experiments used a ratio of 10 ml thioglycolate and 3 ml glycerol. No differences were observed in the hydrolyzing activities of three organisms incubated in both systems.) Each triglyceride was dissolved in hexane (10 mg/ml) and 0.5 ml (containing 5 mg of the triglyceride) was added to each tube of thioglycolate. The hexane was blown off with nitrogen and gentle heating. The tubes were sterilized by autoclave for fifteen minutes at 120° C. (Prior studies demonstrated no breakdown of triglyceride with the sterilization procedure.) Individual bacterial strains were innoculated (50–100 million organisms) into a culture tube containing one of the triglycerides. An appropriate series of control tubes, containing (a) medium and glycerol, (b) medium, glycerol ond organism, and (c) medium, glycerol and triglyceride, was established for each of the 45 triglyceride-microorganism combinations. The tubes were sealed and incubated in an oven for seven days at 35° C. The contents of each tube were agitated daily on a Vortex mixer. Growth of the *C. acnes* organisms was evident in the inoculated tubes about the 3rd day. On the 7th day, each tube was extracted three times with 4 ml portions of chloroform, and the lipids were studied by thin layer chromatography (TLC) and gas liquid chromatography (GLC).

C. Thin layer chromatography (TLC). Conventional techniques of thin layer chromatography were used (4). A slurry of 25 grams of silica gel H (No. 7736, according to Stahl) in 70 ml of glass distilled water was layered (0.25 mm. in thickness) onto 20 × 20 cm glass plates with a Desaga-Brinkmann adjustable applicator. The plates were air dried, then heat activated for 30 minutes at 120° C and allowed to cool. The extracted lipids from each series of four tubes were applied to a single activated chromatographic plate in 300 μg

Received March 3, 1968; accepted for publication August 21, 1968.
Supported in part by funds from U.S. Army Research and Development Command under contract DA-49-193-MD-2184 and by USPHS Training Grant TI-AM-5300 from the National Institute of Arthritis and Metabolic Diseases.
Joyce Stewart gave valuable technical assistance.
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amounts bracketed with appropriate reference standards. The following reference standards (in 20 μg amounts) were used as appropriate: Tricaprylin, tricaprin, trilaurin, tripalmitin, triolein, stearic acid, palmitic acid, dipalmitin, and monopalmitin. The plates were developed for 20 minutes in a glass developing tank (27 × 27 × 7 cm internal dimensions) lined with solvent-saturated Whatman 3 MM paper.

All solvents were redistilled prior to use. The development system used for TLC was hexane-diethyl ether-acetic acid (70:30:1). After development, the plates were air dried, sprayed with dichlorofluorescein or by the method of Jones *et al.* (5) for visualization, viewed and photographed under ultraviolet light. The plates were then exposed to iodine vapors and photographed. Identical thin layer plates, spotted and developed in parallel, were stained with bromocresol green (6) or by the method of Kwapniewski and Sliwiok, as previously modified (7), for the detection of free carboxylic acids.

The free fatty acids were separated from each sample by TLC. Plates were layered with silica gel H (0.25 mm in thickness), predeveloped in chloroform:methanol (9:1, V:V) to remove possible contaminating lipids, air dried, then heat activated for 30 minutes at 120° C and allowed to cool. The extracted lipids were applied to the activated chromatographic plates in 5 to 10 mg samples, flanked by reference standards on the two outside lanes of each plate. The plates were developed as described above, air dried, and the reference standard lanes were sprayed with dichlorofluorescein with the center of the plate screened by plastic film. The silica gel containing the free fatty acids in the central portion of the plate was scraped from the plate into a beaker, extracted three times with 2 ml portions of chloroform, filtered through fat free filter paper and blown to dryness with nitrogen. The methyl esters of the free fatty acids were prepared with BF_3 in methanol (8) and studied by gas liquid chromatography to confirm the presence of the specific fatty acid. GLC conditions were: 20 ft. seamless, stainless steel column, 2.5 mm internal diameter, packed with 8.8% SE-30 on 6.5 grams of silanized, acid-washed Chromosorb W, 80–100 mesh, flow rate ~20 cc per minute of helium carrier gas, at 150° and 205° C. Appropriate reference compounds were used as external standards, and relative carbon numbers were calculated by the method of Woodford and van Gent (9).

RESULTS

This study reports striking differences in the ability of 15 isolates of *C. acnes* to hydrolyze triglycerides *in vitro*. Figure 1 summarizes the results of the study of triglyceride hydrolysis by thin layer chromatography with confirmation of the presence of the specific fatty acid by gas chromatography. Although four strains of *C. acnes* possessed substantial hydrolyzing activity against all three triglycerides, the remaining eleven isolates apparently lacked such diverse ability and split only certain triglycerides. Repeated incubations with several isolates confirmed a consistent pattern of hy-

Organism	Short chain triglyceride*	Tripalmitin	Triolein
C. acnes (C.D.C.)	none evident	?trace	trace
C. acnes (AA # 287)	trace	trace	trace
C. acnes (AA # 271)	yes	yes	yes
C. acnes (AA # 457)	trace	none evident	yes
C. acnes (AA # 459)	trace	none evident	yes
C. acnes (AA # 451)	yes	yes	yes
C. acnes (AA # 452)	none evident	trace	yes
C. acnes (AA # 455)	yes	?trace	yes
C. acnes (AA # 456)	trace	trace	yes
C. acnes (AA # 545)	none evident	none evident	trace
C. acnes (AA # 542)	none evident	none evident	?trace
C. acnes (AA # 544)	yes	yes	yes
C. acnes (AA # 546)	yes	trace	yes
C. acnes (AA # 541)	yes	none evident	yes
C. acnes (AA # 543)	yes	yes	yes

* The first three organisms were incubated with both C_8 and C_{10} triglycerides. The remaining twelve were cultured with C_{12} triglyceride.

FIG. 1. Degree of hydrolysis of three representative triglycerides by 15 isolates of *Corynebacterium acnes in vitro*, as determined by thin-layer chromatography, special TLC staining with bromocresol green and by cupric acetate-hematoxylin, and confirmed by gas chromatography.

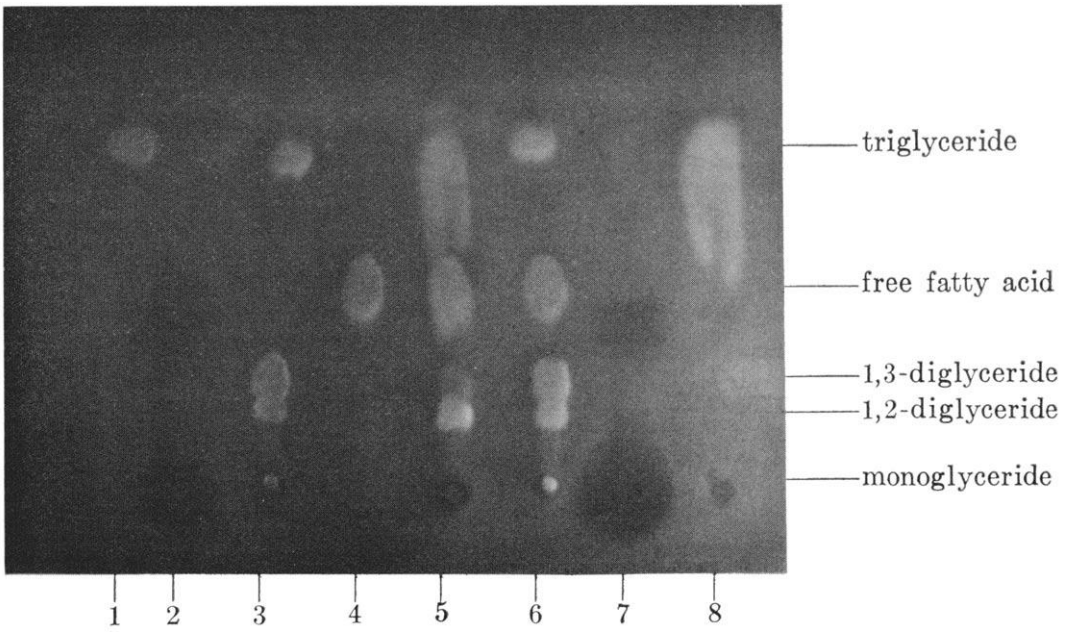

FIG. 2. Thin layer chromatography plate showing hydrolysis of tripalmitin by *C. acnes* ※271; (Lane 1), tripalmitin standard 20 μg; (2) media + organism control, 300 μg; (3) tripalmitin, 1,3-dipalmitin, 1,2-dipalmitin, monopalmitin standards, 20 μg each; (4) palmitic acid standard, 20 μg; (5) tripalmitin + organism, 300 μg (note presence of free fatty acid, 1,3- and 1,2-diglyceride from hydrolysis of triglyceride); (6) tripalmitin, palmitic acid, diglycerides and monoglyceride standards, 20 μg each; (7) media control, 300 μg; (8) media + tripalmitin control, 300 μg.

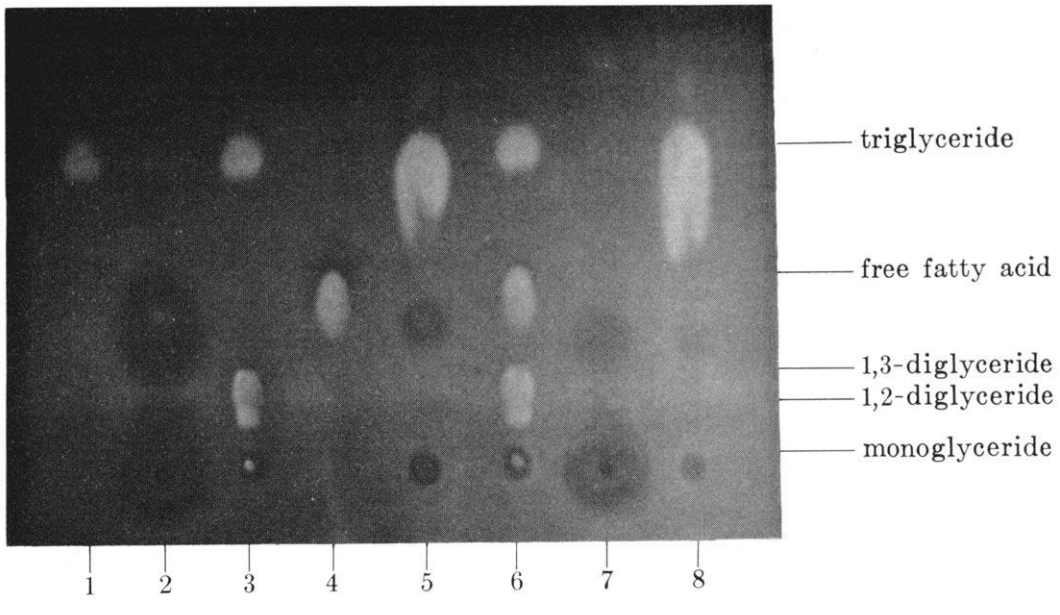

FIG. 3. Thin layer chromatography plate showing failure of *C. acnes* ※287 to hydrolyze significant amounts of triglyceride. Legends for each lane same as Figure 2. Note absence of material in lane 5 corresponding to free fatty acid and diglyceride standards (lanes, 3, 4 and 6).

drolysis for those strains tested. Whether modification of a strain could occur after several weeks or months of subculture was not studied.

Figure 2 is a representative photograph of a thin layer plate showing hydrolysis of tripalmitin by *C. acnes* #271. Lane 5 contained material corresponding to the tripalmitin standard (R_f 0.81), to the free fatty acid (R_f 0.53), and to the 1,3 and 1,2-dipalmitin (R_f 0.32 and 0.24 respectively). Note that the 1,2 isomer of dipalmitin predominates. The spot corresponding to the liberated palmitic acid (lane 5) is bracketed by fatty acid reference standards in lanes 4 and 6. The dark "halo" spots on the plate are extraneous and arise from the media. TLC spots corresponding to the two possible diglyceride fragments of hydrolysis (either 1,3 or 1,2 isomers) were occasionally visualized with tripalmitin, but were seldom seen with triolein and were almost never found with the short chain triglycerides.

Figure 3 is a representative photograph of a thin layer plate showing failure of triglyceride hydrolysis by *C. acnes* #287 (note absence in lane 5 of material corresponding to the free fatty acid standards, or to the 1,3 and 1,2-diglyceride standards). The dark "halo" spots are extraneous and arise from the media.

GLC analysis of the free fatty acids extracted from each sample confirmed the TLC findings. For example, in the tube containing an organism that hydrolyzed tripalmitin, the methyl ester of palmitic acid represented the overwhelmingly dominant peak (>98%) of the fatty acids present. Trace amounts of other fatty acid methyl esters, believed to be of bacterial origin, were usually present with a branched saturated C_{15} acid predominating (10, 11).

The implications of these findings are obvious and perhaps extremely important. Within our working hypothesis, the alternative arises that distinctive strains of *C. acnes* may inhabit the skin of the acne patient, strains with particular abilities to hydrolyze certain triglycerides. These hydrolyzing strains of microorganisms may serve as one of the chief variables in the complex pathogenesis of acne. Experiments to disprove this alternative are in progress.

SUMMARY

Fifteen strains of *Corynebacterium acnes* (*C. acnes*) were incubated individually *in vitro* with three representative triglycerides. Striking differences in ability to split triglycerides were found among the 15 isolates. Four strains, however, demonstrated hydrolyzing ability against all three triglycerides.

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